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Short Review Paper

Isolation and characterisation of lumbrokinase from different species of earthworm

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Abstract

Earthworms have been used in Chinese medicine for past few decades. Earthworm powder was widely used in traditional medicine. The research work is initiated by the study of fibrolytic property in earthworm powder. In traditional Chinese medicine, earthworm powder was used to treat thrombolytic disorder. After that the research work was carried out to know what is responsible for the anti-thrombotic property. Many research works were performed and finally isolate the enzyme responsible for that property and they also find that it is a fraction of protease enzyme .This fraction of enzyme was collectively known as lumbrokinase (LK). There are about 4000 species of earthworm are present in the world. Among them only few species are used as the composting worms. The widely used worms for vermicomposting are Eisenia fetida (red wrigglers), Eudrilus eugeniae (African night crawlers), etc. The intestinal tract of Earthworm was rich in enzymes; these enzymes have numerous therapeutic values. In order to find a drug from natural source; earthworm proteins were used as a source of drug. Lumbrokinase is a protease enzyme has fibrinolytic property so that it was widely used in treating myocardial infraction, thrombosis, DVT. Commercially lumbrokinase was known as Boluoke. Earthworm fibrinolytic enzyme (EFE) also known as Lumbrokinase (LK).

Keywords: Earthworms, Lumbrokinase (LK), Chromatography, SDS PAGE, Fibrinolytic activity, Protease activity.

Introduction

Earthworms are small tube-shaped, legless animal that comes under the kingdom Animalia. There are about 4000 species of earthworm are widely seen in the world. Among them some of the species are widely used for the process of vermicomposting. The species which are widely used in the field of research are *Lumbricus rubellus*[1-3,] *Eisenia fetida*[4,5], *Lampito mauritii*[6]*; Perionyx excavates*[7], *Eisenia Andrei*[8], *Pheretima praepinguis*[9].

Earthworms are used in the Traditional Chinese Medicine (TCM) for past few decades to treat the various thrombotic disorders[1,10,11]. Mostly used species for treating the thrombotic disorder is *Lumbricus rubellus*[12]. Earthworms contain numerous kinds of enzymes in their digestive tract. It is the reason for the digestion of numerous materials by earthworms used in the process of vermicompost. The mid gut region of earthworm contains numerous amount of enzyme and microbes. In Chinese medicine, earthworms are taken orally as powdered form for treating various blood clot, stroke, *etc.* The orally taken earthworm powder shows high positive result when compared to taking a drug. In order to derive a drug from the nature the enzyme isolated from the various earthworm species are used as drug for treating various disease include Myocardial Infraction, DVT(Deep Vein Thrombosis), Ischemic stroke[11], Hypertension, Cancer, Thrombosis, *etc.*

Lumbrokinase is an enzyme which is isolated from the earthworm. It is a fraction of protease enzyme which is mainly responsible for its anti-fibrinolytic property, anti-thrombotic property[10,11]. This enzyme was first isolated from the earthworm species of *Lumbricus rubellus*[1-3]. Later the further researches were carried out in other species includes *Eisenia fetida*[4,5], *Lampito mauritii*[6]*; Perionyx excavates*[7], *Eisenia Andrei*[8,] *Pheretima praepinguis*[9]. Lumbrokinase is otherwise known as EFE (Earthworm Fibrinolytic Enzymes), LK (Lumbrokinase), and Boluoke (Commercial form of LK). In this review we are mainly focused on the isolation and characterization of LK from different species of earthworm.

Collection of earthworm

The Earthworms were collected from the different regions according to their research. Mostly Chinese are doing their research in this field for many years. Their work is mainly focused on the isolation of LK from the *Lumbricus rubellus*[1 3] *E.fetida*[4,5] etc. The collection of worms is mainly from the places like paddy field where the matured earthworms of *Lampito mauritii* are collected[4] ; like that *L.rubelllus* isolated from the Giheung Farmer School, Korea[1,12]; Living earthworm of *P. excavates* were collected from the farms in An Giang province Vietnam[6], *P. praepinguis* were collected from cool, moist, humus-rich soil altitude 500-700 meters from Baoguo Temple to Qingyin pavilion in Mount Emei, Sichuan province,

China[9] and in some researches the earthworm powder was used directly for the enzyme isolation[2].

Isolation of lumbrokinase

Isolation of LK includes various protocols some them isolated it from powder[3] and some from the live earthworm[1,9]. The Fresh earthworms were collected from their areas after that is cleaned with distilled water to remove the soil impurities and weights of worms were noted. Later it was homogenized using 0.1% (w/v) sodium azide[9], or it was homogenized by adding the two volumes of the phosphate buffer (PB) at pH 7.4 [1,4,6,12]. Autolysis was performed by leaving a homogenized sample for 4hrs[1,12], 15hrs[9], placed at 4ºC overnight. In some cases the earthworm were cleaned and tied in an airtight bag and it was placed in sunlight for killing of earthworms and it was freeze dried and then powdered form was obtained; from this powder enzyme was isolated[6]. The homogenized sample was filtered using celite[7] and then again refiltered using 0.42µm membrane filter, then the filtrate was centrifuged at 10,000 rpm for 10 minutes[12], Supernatant was collected[9]. Other than this the different gut regions of earthworms were obtained by narcotized with ice with 10% ethanol[10].

Purification of lumbrokinase

The supernatant obtained from the above process were filtered using 0.42µm membrane filter and then it was placed in a pre-chilled acetone for 2hrs after that precipitate was centrifuged at 5000 rpm for 30 minutes at 4ºC and then it was lyophilized stored at 4ºC[7]. In other method the precipitation was carried by adding 30-60% solid ammonium sulphate to the supernatant and allow it to stand overnight at 4ºC[3,5,10,13,14] and the precipitate was dissolved in phosphate buffer (pH 7.4) then it was dialyzed with dialysis bag and the desalted using a freeze dryer and freeze dried to the powder form known as LK[5,13,14]. In other method the earthworm powder was used directly[4,13,15].

Further purification by chromatographic techniques

The lyophilized powder was resolved in 500ml of 20mM phosphate buffer (pH 7.4). A series of chromatographic techniques were used for purification. Fractions were collected and analyzed by SDS-PAGE after each purification step[1-4,6,7,9,10,12].

Ion Exchange Chromatography: In a diethylaminoethyl - toyopearl 650gum column (4.5*35cm), LK filtrate was stacked and equilibrated with a 20mM phosphate buffer (pH 7.4). After fulfillment of stacking, the adsorbed proteins were eluted at stream pace of 2ml per min with a direct angle of 0-0.5 M NaCl in a similar cushion.

Affinity Chromatography: To remove contaminates, the total of the fractionating was doing using Sephacryl S-200 portion (2.0*75cm). In the wake of stacking, the adsorbed proteins were eluted at a stream pace of 0.3ml per min with a 20mM phosphate support (pH 7.4). Ten ml of each part was accumulated.

Gel Filtration Chromatography: To evacuate the contaminants; fractionating utilizing Sephacryl S-200 segment. In the wake of stacking the sample, chemicals were eluted at stream pace of 0.3ml/min with 20mM of phosphate support. Each portion was gathered and put away at - 4ºC.

Molecular weight determination

The molecular weight was determined by the SDS PAGE. SDS-PAGE was run according to the method of Laemmli using 15% polyacrylamide gel[8]. The sample was stained using Coomassie brilliant blue R-250.The molecular weight was determined by using marker protein[3,6].

Effect of temperature

The caseinolytic assay was performed to determine the optimum temperature. Each fraction of sample was added with 50mM phosphate buffer pH 7.5 and it was placed in the different temperature ranges from 20ºC-70ºC for about 10 minutes. And then enzyme activity was measured at absorbance range of 280nm[7]. In other method the optimum temperature was determined by adding 2ml of LK solution and 1% casein solution and it was incubated for 30minutes at different temperatures the further reaction was stopped by adding 2ml of 10% trichloro acetic acid solution to the mixture. And finally the absorbance was determined at 280 nm and enzyme activity was measured for finding the optimum temperature[9].

Effect of pH: The LK powder was taken and put into a enzyme solution after that 1mlof this solution was added with phosphate buffer of different pH ranges from pH1- pH11. It was incubated at 37ºC for 30 minutes and then absorbance was read at 280nm[1,5,9,14]. The enzyme activity was also determined in various buffers with pH ranges from 2 to 13; 0.1M glycine-HCl buffer for pH 2-3, 0.1M citric acid- Na_2HPO_4 buffer for pH 3-6, 0.1M phosphate buffer for pH 6-8, 0.1M glycine- NaOH buffer for pH 8-11, 0.1M Na_2HPO_4-NaOH buffer for pH 11-12, and 0.1M KCl-NaOH buffer for pH 12-13[3].

Activities of lumbrokinase: Some specific activities of LK includes Fibrinolytic activity[5,13], Proteases activity[3], LK activity[9], Thrombolytic activity[13,16], Amylase activity[10]. The fibrinolytic activity was measured using urokinase as standard by using both plasminogen-rich and plasminogen free fibrin plates. The proteolytic activity was measured using casein plate technique and fibrin plate technique[13]. The LK activity was measured by using following method add 1g of casein in 100 ml of tris HCl buffer (1%) and lysed by heating. And then 2ml of enzyme solution was added with 1ml of substrate solution and finally add 2ml of 10% trichloroacetic acid solution of stop the reaction and incubate it for 30 minutes and filtered. And the

enzyme activity was measured at 280nm. Amylase activity was measured by enzyme solution were added to 450µl 0.02M sodium phosphate buffer, pH 6.9, containing 0.006M NaCl. The reaction was initiated by adding 500µl of a 1% starch solution made in the same buffer. After 3-min incubation at room temperature, the reaction was terminated by the addition of 1.0 ml 3, 5-dinitrosalicylic acid solution and boiled for 5 min. Upon cooling to room temperature, 10ml of water was added, the tubes were vortexed, and the hydrolytic products were estimated by reading the optical densities at 540nm using maltose as the standard.

Properties of Lumbrokinase

The Properties of lumbrokinase includes antimicrobial[4] (anti-bacterial, anti-fungal)[10]; Antidiabetic property[12]. Antidiabetic property- Inhibition of α-Amylase Assay: To 1ml of earthworm extract (100, 200, 400, 800 and 1000µg/mL) add 1ml of enzyme solution and it is incubated at 25ºC for 30 minutes and add 1mL of the 0.5%w/v starch solution and the tube was further incubated at 25°C for 3min. Then 1mL of the color reagent was added and the tube was placed into an 85°C water bath. After 15min, the reaction mixture was removed from the water bath and cooled thereafter, diluted with 9mL distilled water. The absorbance was measured at 540nm. The inhibition of α-amylase (%) was plotted to calculate the IC_{50} value which is concentration of sample (µg/mL) necessary to decrease the absorbance of α-amylase solution by 50%[8,12]. To check the antimicrobial property, the antimicrobial disc was used in other method the zone of inhibition was measured when the erythromycin was used as a control. Antifungal activity was calculated by measuring the zone of inhibition where *candida albicans* is used.

Conclusion

From this review we conclude that different methods were available for the isolation of the enzyme lumbrokinase from the various species of earthworm till date. The application of Lumbrokinase in those days includes treatment of thrombotic disorder, myocardial infraction, etc. But in now a days the further was carried out for the applications of lumbrokinase in the field of treatment of DVT, Hyper coagulation and Cancer treatment.

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